

# Hazard Identification: Efficiency of Short-Term Tests in Identifying Germ Cell Mutagens and Putative Nongenotoxic Carcinogens

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For more than a decade, mutagenicity tests have had a clearly defined role in the identification of potential human mutagens and an ancillary role in the identification of potential human carcinogens. The efficiency of short-term tests in identifying germ cell mutagens has been examined using a combined data set derived from the U.S. Environmental Protection Agency/International Agency for Research on Cancer Genetic Activity Profile (EPA/IARC GAP) and EPA Gene-Tox databases. Our review of these data indicates adequate sensitivity of batteries of *in vitro* short-term mutagenicity tests in identifying germ cell mutagens. The analysis also supports the inclusion of an *in vivo* assay as suggested in proposed regulatory testing guidelines. In the context of carcinogenicity testing, the ability of short-term bioassays to detect genotoxic or mutagenic carcinogens is well established. Such tests are not considered to be as sensitive to nongenotoxic or nonmutagenic carcinogens. However, analyses presented in this report using the EPA/IARC GAP database demonstrate that many putative nongenotoxic carcinogens that have been adequately tested in short-term genetic bioassays induce gene or chromosomal mutation or aneuploidy. Further investigation should reveal whether the mutagenicity of these agents plays an important mechanistic role in their carcinogenicity.

## Introduction

In regulatory practice, mutagenicity tests have a clearly defined, primary role in the identification of potential human mutagens and an ancillary role, when considered with other relevant toxicological information, in the identification of potential human carcinogens. Certain assumptions have been made in the deployment of these tests for the detection of germ cell mutagens. For example, all substances capable of mutating mammalian germ cells are viewed as being able to mutate somatic cells *in vivo*. It is also assumed by many that the great majority of (if not all) substances capable of mutating somatic or germ cells of mammals can be detected with *in vitro* tests. Two *in vitro* tests (one for bacterial mutagenicity and one for chromosome breakage in cultured mammalian cells) are assumed to be sufficient to detect a majority of mutagens. However, the alternative of an additional test (induction of gene

mutations in cultured mammalian cells) is considered by some to be preferable, either as a complement to the two-test battery or in lieu of *in vitro* cytogenetics when carried out using a protocol that permits evaluation of clastogenicity.

Because of the purported sensitivity of *in vitro* systems and the desire to reduce the requirement for animal testing, tests involving mammalian germ cells are not used for screening purposes. It has generally been regarded as prudent, however, to require at least one *in vivo* mammalian mutagenicity test for substances with relatively high human exposure, such as food additives and drugs. The detection of chromosome breakage (aberrations or micronuclei) in rodent bone marrow has been the test of choice.

## How Well Do Short-Term Tests Detect Germ Cell Mutagens?

Mutagenicity tests have now been used for regulatory purposes for more than a decade. In the intervening time, a body of data has accumulated in the literature that permits a retrospective assessment of the performance of short-term tests in the detection of presumptive germ cell mutagens. Therefore, we have produced a combined data set and addressed a number of specific questions regard-

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ing the ability of short-term tests to detect such agents. The results of our analysis indicate an acceptable level of sensitivity for the detection of germ cell mutagens and suggest some ways in which the tests might be deployed in the future.

## How Well Do Short-Term Tests Detect Carcinogens?

As mentioned above, an ancillary role of short-term tests and one that has provoked much controversy (1) is the detection of potential human carcinogens. The utility of short-term tests in the detection of the so-called genotoxic carcinogens has not been seriously questioned. However, the earlier touted overall correlations between mutagenicity in short-term tests and carcinogenicity in rodents have deteriorated since the 1970s. The apparent failure of short-term tests in the detection of carcinogens may be attributed in part to the identification of more and more putative nongenotoxic carcinogens. These agents often induce site-selective carcinogenicity in rodents when administered at or close to the maximum tolerated doses (2). Although short-term tests are not considered to be sensitive to such agents, the overall database for the putative nongenotoxic carcinogens has not been carefully examined.

From a mechanistic perspective and in the context of risk assessment, it is of obvious importance to be able to distinguish between "genotoxic" and "nongenotoxic" carcinogens. In recent years, much attention has focused on the detection and definition of the latter group of agents. Ashby and Tennant (3) recognized that most nongenotoxic carcinogens are not detected in the Ames test and are devoid of alerting (DNA reactive) structural features. They used the combination of positive results in the Ames test and the presence of structural alerts as an index of genotoxicity. Butterworth (4) broadened the definition of a genotoxic agent as "one for which a primary biological activity of the chemical or a metabolite is alteration of the information encoded in the DNA ... point mutations, insertions, deletions or changes in chromosome structure or number." Conversely, Butterworth (4) defined nongenotoxic chemicals as "those that lack genotoxicity as the primary biological activity. While these agents may yield genotoxic events as a secondary result of other induced toxicity, such as forced cellular growth, their primary action does not involve reactivity with the DNA."

In this report we carefully examine a group of putative nongenotoxic carcinogens and classify them on the basis of their mutagenicity per se (ability to induce alterations in DNA structure or content). We have defined criteria for evidence of mutagenicity (and nonmutagenicity) and have applied these criteria to 66 agents that have been cited as nongenotoxic in the published literature (3-7). Three end points (gene mutation, chromosomal aberration, and aneuploidy) were used to evaluate the mutagenicity of these agents. The data clearly demonstrate that many of the putative nongenotoxic carcinogens that have been adequately tested in short-term bioassays induce gene or chromosomal mutations or aneuploidy.

## Methods

In approaching the first analysis (detection of germ cell mutagens), we have considered those substances for which peer-reviewed evidence of germ cell mutagenicity is available. The primary data source was the U.S. Environmental Protection Agency/International Agency for Research on Cancer Genetic Activity Profile (EPA/IARC GAP) computer program (available from M.D.W.), which was supplemented with data from the EPA Gene-Tox database (now available on TOXNET). These peer-reviewed databases (8,9) were constructed using the available published literature and contain a preponderance of positive data.

For our purposes we have assumed that the mutagenicity test results were being used predictively to screen for germ cell mutagens and we therefore present an analysis of the sensitivity of these tests in detecting germ cell mutagens. We have not been able to perform an analysis on the specificity of short-term mutagenicity tests, i.e., their performance with substances that are established as nonmutagenic to mammalian somatic cells or germ cells *in vivo*.

The use of short-term tests in mutagenicity testing is not to define a germ cell mutagen but rather to indicate the potential to cause germ cell mutations. The agent must be shown to reach the germinal tissues to satisfy the presumption that it is a germ cell mutagen. Most published germ cell mutation data have been derived from the evaluation of substances known to be carcinogenic or mutagenic *in vitro* or to be structurally related to such substances.

The data used for the second analysis (detection of putative nongenotoxic carcinogens) are from the GAP database only. A list was compiled of 66 agents cited in the published literature (3-7) as putative nongenotoxic carcinogens. Fifty-three of these agents were present in the GAP database, and 39 of these were represented by five or more test results (Salmonella tester strains considered as individual tests). A table was constructed for the 39 agents emphasizing mutagenicity, i.e., gene mutation, chromosomal aberration, and aneuploidy. Each test result was verified by carefully reviewing the original publication from which data had been extracted. A summary tabular matrix was then prepared based on the strength of the positive data.

The following criteria were applied to the results in assessing the evidence of mutagenicity (or nonmutagenicity) *in vivo* or *in vitro*: a) Evidence of mutagenicity *in vivo* is provided when an agent exhibits activity in at least two tests for one or more of the following end points: gene mutation, chromosomal aberration, or aneuploidy and positive results are obtained in at least one mammalian study *in vivo*; b) Evidence of mutagenicity *in vitro* is provided when an agent exhibits activity in at least two tests *in vitro* for one or more of the above end points. c) Evidence for nonmutagenicity *in vitro* is provided when an agent demonstrates negative results in tests representing all three end points above and includes one mammalian test *in vitro* for gene mutation and one for chromosomal aberration. d) Evidence for nonmutagenicity *in vivo* is

provided when an agent demonstrates negative results in tests representing all three end points above and includes a minimum of two mammalian studies *in vivo*.

## Results and Discussion

### Efficiency of Short-Term Tests in Identifying Germ Cell Mutagens

The germ cell assays that comprise the standard to which other tests are compared are shown in Tables 1 and 2. For analytical purposes, a positive result in any of these tests is considered to define a mammalian germ cell

mutagen. There are obvious limitations to this standard. In many cases only one test has been used, and confirmatory testing is warranted. Moreover, in some instances reviewers may have been overly generous in their acceptance of the data. All mammalian germ cell mutagens for which data from relevant *in vitro* tests were available are shown in Tables 1 and 2. Although protocol adequacy and the quality of the data are major considerations for regulatory purposes, this analysis deals only with the qualitative test results. These data have been peer reviewed and are accepted for the purposes of our present analysis. In a few cases, new data have been added based on the recent published literature.

Table 1. Substances identified as mammalian germ cell mutagens and the test results from the EPA/IARC Genetic Activity Profile database.<sup>a</sup>

Agents	Germ cell mutation <sup>b</sup>									Mammalian cells		Bone marrow
	SLP or SLO	CCC	CGC	CGG	COE	DLM	DLR	MHT	Ames Test	CA	GM	CA or MN
Bleomycin			+					+	+	+	+	+
Cisplatin		+	+			-			+	+	+	+
Cyclophosphamide		+	+	+	+	+	+	+	+	+	+	+
Ethylene oxide		+				+	+	+	+	+	+	+
Myleran	-		-			+	+		+	+	+	+
Thiotepa		+		+	+	+		+	+	+	+	+
Adriamycin			+			+			+	+	+	+
Aflatoxin B <sub>1</sub>			-			+			+	+	+	+
BCNU		+	?	-					+	+	+	+
Benzo[a]pyrene	-					+			+	+	+	+
Nitrogen mustard						+	<sup>b</sup>		+	+	+	+
Potassium dichromate						+			+	+	+	+
6-Mercaptopurine						+	?		+	+	+	+
Chloramphenicol		+		+		-			-	+	+	+
Diethyl sulfate					+	+			+	+	+	NA
Procarbazine HCl	+					?		-	+	NA	+	+
Triaziquone					+	+		+	+	+	NA	+
Azathioprine						+			+	+	NA	+
CCNU							+		+	+	+	NA
Chlorambucil	+								+	+	+	NA
Chloroprene						+	+		+	NA	-	+
Acetaldehyde					+				+	+	NA	NA
N-Butyl glycidyl ether						+			+	NA	NA	+
Dibromochloropropane						-	+		+	+	NA	NA
Furosemide		+							-	+	+	NA
Mercuric chloride				-	-		+		-	+	+	NA
Chloromethane							+		+	NA	+	NA
Diazepam						+			-	-	NA	+
Saccharin, sodium				-		+			-	+	-	-
Trimustine						+			NA	NA	+	NA
Ethanol				-	+	+	+		-	+	-	-
Norethisterone acetate						+			-	-	NA	NA
Tris(2-chloroethyl) phosphate							+		-	NA	-	NA
DDT			+			-	+		-	?	-	?

Abbreviations: Mouse specific locus tests: SLP, postspematogonial and SLO, other stages; chromosomal aberrations *in vivo*: CCC, spermatocytes treated and observed; CGC, spermatogonia treated and spermatocytes observed; CGG, spermatogonia treated and observed, and COE, oocytes or embryos; dominant lethal tests: DLM, mice; DLR, rats; MHT, mouse heritable translocation test; CA, chromosomal aberrations; GM, gene mutation; MN, micronuclei.

<sup>a</sup>Positive (+), negative (-), and conflicting (?) test results are indicated; NA, not available.

<sup>b</sup>Germ cell results have been updated for chlorambucil (10) and nitrogen mustard (11).

Table 2. Substances identified as mammalian germ cell mutagens and the test results from the Gene-Tox Program.<sup>a</sup>

Agents	Germ cell mutation							Ames Test	Mammalian cells		Bone marrow
	SLT	CCC	CGC	CGG	COE	DLT	MHT		CA	GM	CA or MN
1-Ethyl-1-nitrosourea	+						+	+	+	+	+
Ethyl methanesulfonate	+					+	+	+	+	+	+
Methyl methanesulfonate	+	+			+	+	+	+	+	+	+
Mitomycin C	+	+			+	+	+	+	+	+	+
Methotrexate					+			+	+	+	+
1-Methyl-1-nitrosourea	+					-	+	+	+	+	NA
Captan						+	-	+	NA	+	+
Hycanthone methane-sulfonate	-					+		+	NA	+	+
Trimethyl phosphate						+		+	+	NA	+
Isopropyl methanesulfonate					+	+	+	NA	NA	+	+
Triethylenemelamine	+	+			+	+	+	NA	+	NA	+
TEPA						+	+	+	+	NA	NA
Hexametapol						+		NA	-	+	+
N-Ethyl nitrosourethane	+							+	NA	NA	NA
Fotrin						+		NA	+	NA	NA
2-OH-nitrosourea	+							+	NA	NA	NA
METEPA						+		NA	NA	NA	+
Nitrogen mustard N-oxide						+		+	NA	NA	NA
Streptonigrin					+			NA	+	NA	NA
Triflupromazine						+		NA	+	NA	-
Acrylamide	+ <sup>b</sup>		+	+				-	NA	NA	+ <sup>b</sup>
Ergotamine tartrate						+		-	NA	NA	NA
Octyl adipate						+		-	NA	NA	NA

Abbreviations: SLT, mouse specific locus tests; chromosomal aberrations *in vivo*: CCC, spermatocytes treated and observed; CGC, spermatogonia treated and observed; CGG, spermatogonia treated and observed; COE, oocytes or embryos; DLT, dominant lethal tests; MHT, mouse heritable translocation test; CA, chromosomal aberrations; GM, gene mutation; MN, micronuclei.

<sup>a</sup>Positive (+), negative (-), and conflicting (?) test results are indicated; NA, not available.

<sup>b</sup>Acrylamide results are updated from the recent literature (12-14).

As shown in Figure 1, results from the combined GAP and Gene-Tox databases are as follows: Of 56 germ cell mutagens, 52 had been subjected to bacterial mutagenicity and/or *in vitro* mammalian cell chromosome breakage assays, and of these 87% gave a positive result. Evaluation of those substances for which both tests were done showed that 32 out of 34 mutagens were detected. The two substances not identified as mutagens by this battery (diazepam and norethisterone acetate) depended on a single mammalian germ cell test (dominant lethal) for their inclusion in the germ cell mutagen list. These agents are not well studied but, like ethanol (negative in a wide range of mutagenicity assays), have been shown to cause malsegregation of chromosomes (aneuploidy), which could account for the dominant lethal results.

If a test for mammalian cell mutagenesis is added to the above two tests, the following results are obtained. Of the germ cell mutagens tested in one or more of the three tests, 87% were identified as mutagens, i.e., three additional positives were identified. Analysis of substances for which all 3 tests were performed showed all 26 were identified as mutagens. Although these figures are marginally higher than those for the two-test battery, the difference reflects the more restricted number of compounds tested.

Because one of the most widely used gene mutation assays (selecting for thymidine kinase deficiency in L5178Y mouse lymphoma cells) also responds to chromosome deletions formed by chromosome breaking agents, there is in principle no reason why a mammalian gene mutation assay such as this should not be used to replace the *in vitro* cytogenetics and mammalian cell mutagenesis assays described above. This combination, i.e., bacterial mutation test and mammalian cell gene mutation assay (with a protocol for assessment of chromosome breakage), demonstrates the following results. As shown in Figure 1, of 52 substances tested in either one or both of tests just described, 42 were positive. Of the 34 that were tested in both tests, 30 were identified as mutagens. These figures are similar to those for the previous two test batteries, which suggests (albeit based on a relatively small data set) that the use of the cheaper mammalian cell gene mutation assay as an alternative to the assay for mammalian cell chromosome breakage deserves consideration. The four germ cell mutagens missed were DDT, sodium saccharin, ethanol, and tris(2-chloroethyl) phosphate (Fig. 1).

Given that a clear positive result in any of the tests mentioned above is normally enough to warrant a requirement for an *in vivo* assay, is it necessary to do both *in vitro* assays if the first one done is positive and, if not, which

assay should be done first? As shown in Figure 1, there is no doubt that the mammalian cell chromosome breakage assay identifies more accurately the germ cell mutagens (93%) than does the bacterial mutation assay (75%). [The mammalian cell gene mutation assay is also high (86%)]. The mammalian cell chromosome breakage test, although the more sensitive, is also more expensive. A case could be made for performing the bacterial mutagenicity test first, and only performing a mammalian cell chromosome breakage assay if a negative result is obtained:

In view of the fact that there are bacteria in the gut of mammals and that the liver may be able to carry out metabolic transformations that cultured cells cannot, even with S-9 microsomal fraction, it seems prudent at the present time to subject bacterial mutagens to *in vivo* testing. This would normally involve looking for the induction of chromosome breakage in cells of the rodent bone marrow, either scoring micronuclei or broken chromosomes at metaphase. The rationale for this is that not all substances that are mutagenic *in vitro* are able to express significant mutagenic activity in the whole animal. Bone marrow assays are preferred because they are widely used and reliable and because it is reasoned that a mutagen capable of reaching the gonads ought also to reach the bone marrow. We may therefore ask whether the data show that germ cell mutagens are a subset of bone marrow mutagens.

As shown in Figure 1, of 36 germ cell mutagens tested in a bone marrow assay (either aberrations or micronuclei),

33 were identified as mutagens. The 3 not identified were ethanol, sodium saccharin, and trifluoromazine. Adler and Ashby (15) found the evidence for germ cell mutagenicity of sodium saccharin and trifluoromazine questionable. Sodium saccharin is usually included in lists of reference nongenotoxic carcinogens. Ethanol (also reviewed by these authors) may act by inducing aneuploidy, which the bone marrow assays were not designed to detect. Obe and Anderson (16) state that ethanol shows strain dependent differences in the bone marrow assays. Overall, the data are consistent with the view that germ cell mutagens are probably not unique and that some of the bone marrow assays in the database were not performed using current protocols and therefore were not of adequate power (17). It should be noted that the bone marrow assay detects not only substances shown to cause chromosomal damage in rodent germ cells, but also those known to induce gene mutations in the specific locus coat color test. This reinforces the generalization that specific gene (as distinct from chromosomal) mutagens are rare. Thus, there is some security in using an *in vivo* chromosome breakage assay for presumptive germ cell mutagens. Adler and Ashby (15) concluded that "the general observation that rodent germ cell mutagens are also genotoxic in somatic cells *in vivo* remains valid." We would only add the comment that the problem of strain variability among rodents contributes to the discrepancies with trifluoromazine and ethanol and is an underlying problem in all of toxicology.

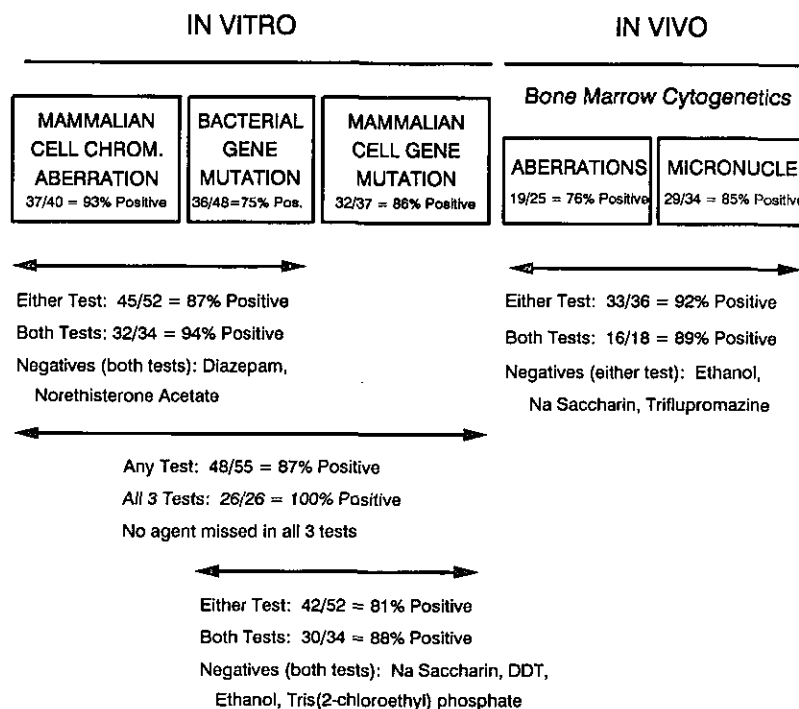


FIGURE 1. Test performances are given for the germ cell mutagens from the combined EPA/IARC GAP and Gene-Tox databases. Performance is indicated by the fraction of agents with positive test results divided by the number of agents tested and is expressed also as the percentage positive. Combinations of short-term tests are illustrated by the range of arrows.

## Evidence of Mutagenicity of Putative Nongenotoxic Carcinogens

Using the criteria for evidence of mutagenicity presented in Methods, we were not successful in segregating the putative nongenotoxic carcinogens into only four categories. Thus, the word "limited" was added to identify those agents that met most of the stated criteria. Figures 2 and 3 present a summary of the short-term test results for the 39 agents that could be grouped according to the resulting six categories of evidence. The qualitative data for these agents are presented according to the three genetic end points; gene mutation, chromosomal aberration, and aneuploidy. A miscellaneous category is included for data on cell transformation and sister-chromatid exchange. Test systems are identified by three-letter code words (see appendix for definitions) and *in vitro* tests are separated from *in vivo* tests for each end point. In general, the agents investigated fall into two groups: 23 agents showing evidence of mutagenicity (9 of which are included in the limited evidence category), and 16 agents showing evidence of nonmutagenicity, (14 of which show limited evidence). It should be noted that essentially all of these compounds are negative in the Ames test (exceptions are chlorodibromomethane with conflicting results, and nitriolotriacetic acid [NTA], not tested). Furthermore, structural alerts are identified only for *p*-chloro-*o*-toluidine,

aniline, chlorodibromomethane, and carbon tetrachloride (3,18).

**Evidence of Mutagenicity.** The positive data for the seven agents that display evidence of mutagenicity *in vivo* (Fig. 2) represent primarily chromosomal effects (chromosomal aberrations and/or micronuclei in mouse bone marrow) or, in the case of diethylstilbesterol (DES) and NTA, induction of aneuploidy. Although induction of aneuploidy does not necessarily indicate that a chemical has interacted directly with DNA, clearly, a structural genetic alteration (alteration in DNA content) has resulted from chemical treatment. Results of cell transformation and sister chromatid exchange assays generally strengthen the case for classifying these compounds as a group.

As with the previous category, the seven agents displaying evidence of mutagenicity *in vitro* exhibit a large body of positive data for chromosomal mutation; all except asbestos appear to be eukaryotic gene mutagens as well. Three agents, malonaldehyde, 2,4-D, and *p,p'*-DDE, are gene mutagens in *Drosophila* and in mammalian cells in culture but not in bacteria. The inclusion of all seven agents within the category of *in vitro* evidence is supported by positive data from cell transformation and sister chromatid exchange assays.

Agents listed in the category of limited evidence for mutagenicity are classified as such because of conflicting results or, more often, weak positive responses, frequently

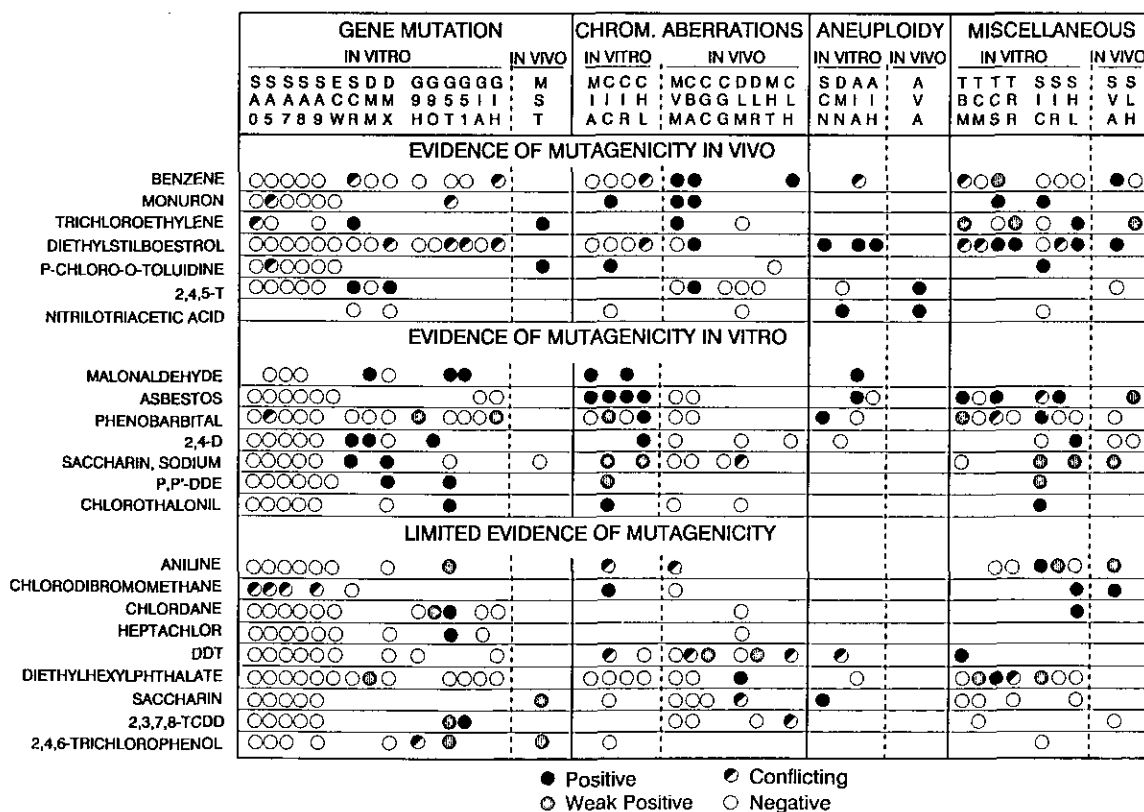


FIGURE 2. Summary of short-term test results for 23 putative nongenotoxic carcinogens displaying evidence of mutagenicity.

	GENE MUTATION				CHROM. ABERRATIONS				ANEUPLOIDY		MISCELLANEOUS			
	IN VITRO		IN VIVO		IN VITRO		IN VIVO		IN VITRO	IN VIVO	IN VITRO		IN VIVO	
	SSSSSE	SDD	GGGGGG	M	MCCC	MCCDDMC	SDAA	A	TTTT	SSS	SS	SS	SS	SS
	AAAAAC	MM	995511	S	111H	VBGGLLHL	CM11	V	BCCR	11H	VL	VL	VL	VL
	05789	WRMX	HOT1AH	T	ACRL	MACGMRT	NNAH	A	MMSR	CRL	AH	AH	AH	AH
LIMITED EVIDENCE OF NONMUTAGENICITY IN VITRO														
ETHINYLESTRODIOL	○ ○ ○ ○ ○	○ ○ ○			○ ○						○ ○ ○			
DECABROMODIPHENYL OXIDE	○ ○ ○ ○	○			○						○			
1,4-DIOXANE	○ ○ ○ ○ ○	○			○				○		○			
NORETHISTERONE ACETATE	○ ○ ○ ○ ○				○				○	○				
PENTACHLOROETHANE	○ ○ ○ ○	○			○ ○						○			
1,1,1,2-TETRACHLOROETHANE	○ ○ ○ ○	○			○						○			
LIMITED EVIDENCE OF NONMUTAGENICITY IN VIVO														
PBB	○ ○ ○ ○ ○	○ ○ ○ ○			○ ○ ○ ○									
17B-OESTRADIOL	○ ○ ○ ○ ○	○ ○			○ ○	○ ○			○				○	
HEXACHLOROCYCLOHEXANE	○ ○ ○ ○ ○	○ ○			○ ○	○ ○	○							
HEXACHLOROBENZENE	○ ○ ○ ○ ○	○ ○			○ ○	○ ○	○							
MELAMINE	○ ○ ○ ○ ○	○			○	○							○	
CARBON TETRACHLORIDE	○ ○ ○ ○ ○	○ ○			○ ○	○ ○			○				○	
RESERPINE	○ ○ ○ ○ ○				○	○								
TETRACHLOROETHYLENE	○ ○ ○ ○ ○	○ ○			○ ○	○ ○					○	○		○
EVIDENCE OF NONMUTAGENICITY IN VIVO														
PCB	○ ○ ○ ○ ○ ○				○ ○ ○ ○ ○				○		○			
MCPA	○ ○ ○ ○ ○ ○	○ ○			○ ○ ○ ○ ○				○		○	○	○ ○	

● Positive    ○ Conflicting  
 ○ Weak Positive    ○ Negative

FIGURE 3. Summary of short-term test results for 16 putative nongenotoxic carcinogens displaying evidence of nonmutagenicity.

for two or more end points. Agents in this category should not be considered to pose a serious mutagenic hazard.

**Evidence of Nonmutagenicity.** Only 2 agents, polychlorinated biphenyls (PCB) and (4-chloro-2-methylphenoxy)acetic acid (MCPA) (shown at the bottom of Fig. 3), clearly conform to the criteria for evidence of nonmutagenicity *in vivo*. Negative test results representing all three end points are reported for these 2 compounds including two or more *in vivo* mammalian studies for chromosomal aberrations. The remaining compounds are listed under the categories of limited evidence for nonmutagenicity *in vitro* (6 agents) and limited evidence of nonmutagenicity *in vivo* (8 agents). The limitations reflect inadequate testing for the three genetic end points. The agents showing evidence of nonmutagenicity are not as thoroughly tested as are those agents with evidence of mutagenicity. Nonetheless, these 16 compounds may be considered as nonmutagenic carcinogens based on the data available in the EPA/IARC GAP database.

A summary of NTP and IARC evaluations of animal and human carcinogenicity classifications for the 39 putative nongenotoxic carcinogens presented in this paper is given in Figure 4. The NTP evaluations are presented by species and sex together with the number of the NTP Technical Report from which the information was obtained. The IARC evaluation represents animal and human studies as well as an overall evaluation of the carcinogenic potential for humans. Possible mechanisms of carcinogenesis are also indicated (refer to Conclusions).

Based on IARC's evaluation of 22 of the remaining 27 agents for which there was insufficient mutagenicity data

for inclusion in this analysis, 17 are "not classifiable as to carcinogenicity in humans" and the remainder are considered as "possibly carcinogenic to humans." Limited evidence of mutagenicity and carcinogenicity for these agents suggests that it may be inappropriate to classify them as nongenotoxic carcinogens at present.

Overall, when comparing agents with evidence of mutagenicity to those showing evidence of nonmutagenicity (Fig. 4), it is difficult to find any remarkable distinctions between the two groups based on the weight-of-evidence for carcinogenicity. Closer examination of the NTP carcinogenicity data yields similar results. Figure 5 shows a graphic representation of the data organized by tumor site, species, and sex. The first column indicates evidence of mutagenicity or nonmutagenicity. Again, no clear relationship was apparent between the carcinogenicity of these agents in any site and their mutagenicity in short-term tests. The data do show, however, that 7 of 11 mouse hepatocarcinogens tested are nonmutagenic, and this observation warrants further investigation.

Finally, attempts were made to assess the relationship between mutagenicity and potential mechanisms of carcinogenesis (Fig. 4). Various kinds of biological activity have been associated with the nongenotoxic carcinogens including enhanced cell proliferation (resulting from cytotoxicity or mitogenesis), hormonal changes, tumor promotion, and peroxisome proliferation. Six peroxisome proliferators (7) were identified among the agents evaluated in this study. Four of these, (2,4,5-trichlorophenoxy)acetic acid (2,4,5-T), (2,4-dichlorophenoxy)acetic acid (2,4-D), diethylhexylphthalate, and trichloroethylene

MECH. <sup>a</sup>	COMPOUNDS	NTP <sup>b</sup>					IARC <sup>c</sup>		
		TR#	MR	FR	MM	FM	H	A	EVAL. MONO.
EVIDENCE OF MUTAGENICITY IN VIVO									
A	BENZENE	289	●	●	●	●	●	●	1 Suppl. 7
	MONURON	266	●	○	○	○	●	●	3 Vol. 53
	TRICHLOROETHYLENE	243	●	○	○	○	●	●	3 Suppl. 7
D	DES						●	●	1 Suppl. 7
A	P-CHLORO-O-TOL <sup>d</sup>	165	○	○	●	●	●	●	2A Vol. 48
B	2,4,5-T						○	●	2B Suppl. 7
	NTA	6	●	●	●	●	●	●	2B Vol. 48
EVIDENCE OF MUTAGENICITY IN VITRO									
C,E	MALONALDEHYDE <sup>d</sup>	331	●	●	○	○	●	●	3 Vol. 36
	ASBESTOS	285	●	○			●	●	1 Suppl. 7
	PHENOBARBITAL						○	○	2B Suppl. 7
A	2,4-D						○	○	2B Suppl. 7
B	SACCHARIN, SODIUM						○	●	2B Suppl. 7
	P,P'-DDE	131	○	○	●	●			Vol. 53 <sup>e</sup>
	CHLOROTHALONIL	41	●	●	○	○	○	●	3 Suppl. 7
LIMITED EVIDENCE OF MUTAGENICITY									
	ANILINE <sup>d</sup>	130	●	●	○	○	●	●	3 Suppl. 7
	CHLORODIBROMOMETHANE	282	○	○	○	○	●	●	3 Vol. 52
	CHLORDANE	8	○	○	●	●	●	●	2B Vol. 53
	HEPTACHLOR	8	○	○	●	●	●	●	2B Vol. 53
	DDT	131	○	○	○	○	●	●	2B Vol. 53
A	DEHP	217	●	●	●	●	●	●	2B Suppl. 7
B	SACCHARIN						○	●	2B Suppl. 7
E	2,3,7,8-TCDD	209	●	●	●	●	●	●	2B Suppl. 7
	2,4,6-TRICHLOROPHENOL	155	●	○	○	○	○	●	2B Suppl. 7
LIMITED EVIDENCE OF NONMUTAGENICITY IN VITRO									
C,D	ETHINYLOESTRADIOL						●	●	1 Suppl. 7
	DECABROMODIPHENYL OX.	308	●	●	○	○	●	●	3 Vol. 48
	1,4-DIOXANE	80	●	●	●	●	●	●	2B Suppl. 7
D	NORETHISTERONE ACET.						○	●	2B Suppl. 7
B	PENTACHLOROETHANE	232	●	○	○	○	○	○	3 Vol. 41
A	1,1,1,2-TETRACHLOROETHANE	237	●	○	○	○	○	○	3 Vol. 41
LIMITED EVIDENCE OF NONMUTAGENICITY IN VIVO									
C	PBB	244	●	●	●	●	○	●	2B Suppl. 7
D	17β-OESTRADIOL						●	●	1 Suppl. 7
C,E	HEXACHLOROCYCLOHEXANE	14	○	○	○	○	○	●	2B Suppl. 7
	HEXACHLOROBENZENE						○	●	2B Suppl. 7
	MELAMINE	245	●	○	○	○	○	○	3 Vol. 39
B	CARBON TETRACHLORIDE						○	●	2B Suppl. 7
	RESERPINE	193	●	○	○	○	○	○	3 Suppl. 7
B	TETRACHLOROETHYLENE	311	●	○	○	○	○	●	2B Suppl. 7
EVIDENCE OF NONMUTAGENICITY IN VIVO									
A	PCB						○	●	2A Suppl. 7
	MCPA						○	○	2B Suppl. 7

FIGURE 4. Summary of NTP and IARC carcinogenicity evaluations. Compounds are organized according to the evidence of mutagenicity or nonmutagenicity as shown in Figures 2 and 3.

<sup>a</sup>Potential mechanism of action, A: peroxisome proliferation; B: cell proliferation (cytotoxic); C: cell proliferation (mitogenic); D: hormonal action; E: tumor promotion.

<sup>b</sup>Headings are, TR#: Technical Report No. MR: male rats; FR: female rats; MM: male mice; FM: female mice; carcinogenicity evaluation, ● positive; ○ limited; ● equivocal/insufficient; ○ negative. [National Toxicology Program Technical Reports, NTP, Research Triangle Park, NC (27)].

<sup>c</sup>Headings are, H: human data; A: animal data; EVAL: overall evaluation; MONO: IARC Monographs volumes. IARC overall evaluation, 1: carcinogenic to humans; 2A: probably carcinogenic to humans; 2B: possibly carcinogenic to humans; 3: not classifiable as to its carcinogenicity to humans. [International Agency for Research on Cancer, Lyon, France (28)].

<sup>d</sup>NTP data are from studies with *p*-chloro-*o*-toluidine, HCl; malonaldehyde, Na salt; and aniline, HCl.

<sup>e</sup>IARC summarized the carcinogenicity data for DDT and its metabolites but only evaluated DDT.

displayed evidence of mutagenicity (Fig. 2), while MCPA and 1,1,1,2-tetrachloroethane did not (Fig. 3). Similarly, two of five cytotoxins (4), NTA and sodium saccharin, showed evidence of mutagenicity (Fig. 2), but carbon tetrachloride, pentachloroethane, and tetrachloroethylene did not (Fig. 3). One of three mitogens (4), phenobarbital (Fig. 2), was considered mutagenic while polybrominated biphenyls (PBB) and hexachlorocyclohexane showed limited evidence of nonmutagenicity *in vivo* (Fig. 3). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), identified as a promoter that appears to act through specific receptors (4), showed limited evidence of mutagenicity (Fig. 2). Three hormones (ethinyloestradiol, norethisterone acetate, and

17β-estradiol) are represented among agents showing limited evidence of nonmutagenicity *in vivo* (Fig. 3) and one (diethylstilbestrol [DES]) is listed with agents showing evidence of mutagenicity *in vivo* (Fig. 2). Thus, multiple putative mechanisms of carcinogenicity are suggested for the mutagenic as well as the nonmutagenic carcinogens represented in Figures 2 and 3, respectively.

## Conclusions

Dearfield et al. (19) have discussed recently the testing approaches used by the EPA Office of Pesticide Programs (OPP) and Office of Pollution Prevention and Toxics



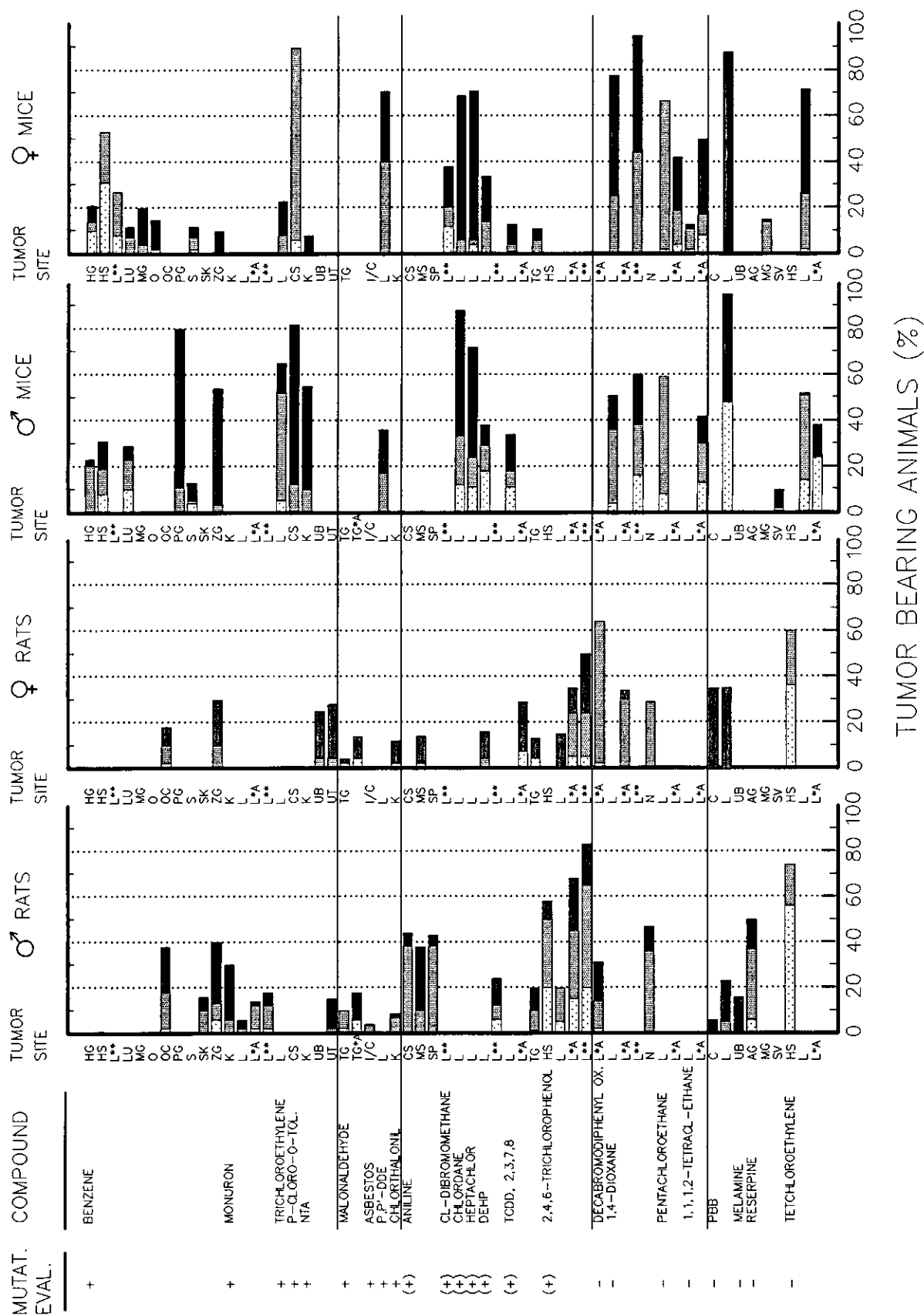
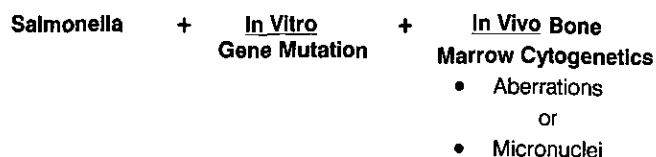


FIGURE 5. Carcinogenicity data from NTP reports for 24 chemicals sufficiently tested for mutagenicity are illustrated in this graph. The percentage of animals bearing tumors is presented for the control (dotted boxes); low-dose (light-shaded boxes); and high-dose groups (dark-shaded boxes) (note: bars are superimposed so each has its origin at 0). The data are organized by species, sex, and tumor site. The column labeled "Mutat. Eval." indicates +, mutagenicity; (-), limited evidence of mutagenicity; or -, limited evidence of nonmutagenicity from short-term tests. Tumor sites are identified as follows: AG, adrenal gland; C, cholangioma; CS, circulatory system; HG, Harderian gland; HS, hepatocellular system; I/C, intestine/colon; K, kidney; L, liver; L\*, liver adenomas; L\*\*, liver carcinomas and adenomas; LU, lung; MG, mammary glands; MS, multiple sites; N, nose; O, ovary; PG, preputial gland; S, stomach; SK, skin; SP, spleen; SV, seminal vesicle; TG, thyroid gland; TG\*, A, thyroid gland adenomas; UB, urinary bladder; and ZG, Zymbals gland.



*In Vitro* Gene Mutation (choice):

- (a) Mouse lymphoma L5178Y cells, TK locus, small and large colonies
- (b) Chinese hamster ovary cells, strain AS52
- (c) Chinese hamster ovary cells or lung fibroblasts with the appropriate *in vitro* test for clastogenicity

FIGURE 6. EPA's Office of Pesticide Program's mutagenicity test guideline.

(OPPT). The scheme outlined in Figure 6 represents the proposed OPP mutagenicity test guideline. The first two components in the guideline essentially represent the three-test battery previously discussed (Fig. 1). The *in vivo* tests in the OPP screening battery include the same bone marrow assays (aberrations or micronuclei) mentioned above.

Under the OPPT testing scheme shown in Figure 7, the initial battery is essentially the same as that of the OPP as discussed above. Most chemicals would require no further testing if found negative in all three of the tests in the first tier. However, subsequent germ cell tests or cancer bioassays may be warranted based on other information such as human exposure, compelling chemical structure-activity relationships, or other factors of concern. Positive test results in the initial tier would trigger further testing in the second tier; additional positive results in the second

tier tests would trigger the appropriate germ cell tests. The results of the present analysis lend support to the regulatory strategy for mutagenicity testing that has been outlined by the EPA. There are additional implications in terms of carcinogenicity testing as discussed below.

In any sizeable series of chemicals subjected to mutagenicity testing, two groups of agents will be found that are mutagenic *in vitro* but that are not mutagenic to mammalian germ cells. The first group are those that produce chromosomal damage in the rodent bone marrow. These (or their active metabolites) may not penetrate to the gonads but must be presumed to have the potential to produce chromosomal damage in somatic tissues *in vivo*. The regulatory position is that, in the absence of evidence to the contrary, it is prudent to assume that any chemical capable of causing mutations *in vivo* in mammals is a potential carcinogen. In the light of this view, it was in the past rare to proceed to a mammalian germ cell assay because the available mutagenicity information was generally considered sufficient for regulation based on carcinogenic potential. The new regulations mentioned above will require further evaluation of mutagenic potential per se.

The second group of compounds is more difficult to deal with. They are those that are positive *in vitro* but that do not produce detectable chromosome damage in the bone marrow. This group will include some that are not absorbed, are not activated *in vivo*, or are immediately inactivated or detoxified. These may be presumed not to present a mutagenic or genotoxic hazard in animals or in man. Others, however, will be active, but their active species do not reach the bone marrow. The gut and the liver are clearly potential targets for such agents. Most of the evidence for such "local" activity pertains to direct-acting alkylating agents. Ashby (20) has pointed out how unwise it is to neglect these agents because their genotoxicity is not systemic. The typical regulatory position is that further work is needed to provide reassurance that there is no mutagenic or genotoxic action in the gut or liver. The nature of such work is determined on a case-by-case basis. The techniques currently available often leave much to be desired in terms of reproducibility, and many have not been adequately validated. Some of the most difficult problems in genetic toxicology occur in this area, and it is one in which high hopes are held for the future application of recently developed assays with transgenic mice.

Another problem area in genetic toxicology is that of interpreting short-term tests results for putative non-genotoxic carcinogens with regard to hazard identification and risk assessment. It is difficult to ignore the positive mutagenicity test results reported for many of the agents discussed in this report, even in light of evidence that the carcinogenicity of some of them may be mediated primarily through nongenotoxic mechanisms (4). Unless an agent has been tested for each of the three broad categories of DNA alterations, i.e., gene mutation, chromosomal aberration and aneuploidy, it is inappropriate to conclude that the compound is nonmutagenic. Similarly, the strength of the evidence for carcinogenicity must also be carefully evaluated. For example, malonaldehyde is obviously mutagenic *in vitro*, but the evidence of carcino-

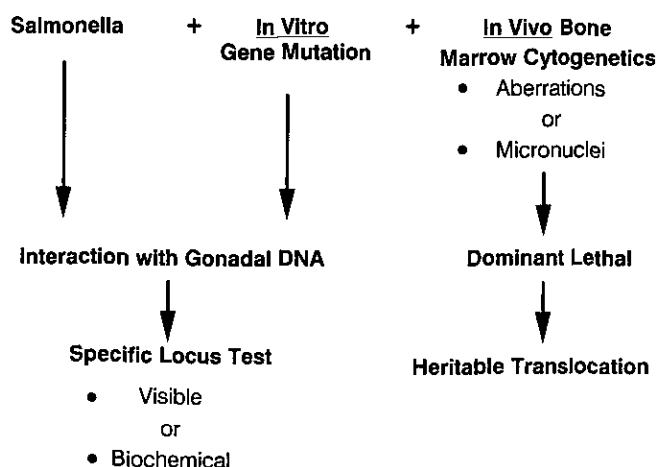


FIGURE 7. The Office of Pollution Prevention and Toxics mutagenicity test scheme.

genicity is weak. Thus, IARC lists malonaldehyde as "not classifiable as to its carcinogenicity in humans," and the NTP data show a low incidence of tumor formation in the rat thymus gland (Fig. 4). Ashby and Morrod (2) note that many nongenotoxic agents induce selective carcinogenicity in rodents after treatments at or close to the maximum-tolerated doses and that the activity of these compounds in humans cannot always be assumed. Sodium saccharin induces bladder tumors in rats administered 5% saccharin in the diet (21), a dose much higher than the levels present in normal human diets. The evaluation of human risks associated with exposures to such compounds must be performed on a chemical-by-chemical basis considering not only the strengths and weaknesses of the mutagenicity data but of the carcinogenicity data as well.

More research is needed on the mechanisms of action of the putative nongenotoxic carcinogens to ensure proper evaluation of these agents in the context of quantitative risk assessment. Many biological activities may or may not be relevant to the ultimate mechanisms of carcinogenicity. For example, a number of putative nongenotoxic rodent hepatocarcinogens have been shown to cause peroxisome proliferation (7). Although peroxisome proliferation has been demonstrated in most rodent species tested, nonhuman primates are only weakly responsive, suggesting that humans are at limited risk of carcinogenic effects due to this phenomenon (22-25). Other compounds, such as carbon tetrachloride, chloroform, tetrachloroethylene, pentachloroethane, and sodium saccharin, appear to produce tissue damage by cytotoxicity resulting in regenerative hyperplasia (26). The specific mechanisms involved or the conditions under which these agents produce their effects, as mentioned earlier in the case of sodium saccharin, may have little if any relationship to human cancer risk.

In summary, the efficiency of current procedures for screening mutagens has been examined using a combined data set derived from the EPA/IARC GAP and Gene-Tox databases. Our review of these data indicates that the sensitivity of the batteries of *in vitro* short-term mutagenicity tests currently used to identify potential germ cell mutagens is approximately 90%. Some possible improvements in the deployment of such tests, such as the inclusion of a mammalian assay for induction of aneuploidy, are worthy of further exploration. However, the data affirm the utility of short-term tests in hazard identification and support current regulatory testing strategies.

In the context of carcinogenicity testing, the ability of short-term tests to detect genotoxic or mutagenic carcinogens is well established. Such tests are not viewed as sensitive to nongenotoxic or nonmutagenic carcinogens. Analyses presented in this report (Fig. 2) using the EPA/IARC GAP database, however, demonstrate that many putative nongenotoxic carcinogens that have been adequately tested in short-term genetic bioassays induce gene or chromosomal mutation or aneuploidy. The ability of compounds to induce such alterations in DNA structure or content cannot be ignored as work continues toward understanding the mechanisms of carcinogenesis and potential cancer risks for human populations. Further

investigation of the agents listed in Figure 2 should reveal whether their mutagenicity plays an important mechanistic role in their carcinogenicity.

## Appendix

### Short-Term Test Code Definitions

Test code	Definition
AIA	Aneuploidy, animal cells <i>in vitro</i>
AIH	Aneuploidy, human cells <i>in vitro</i>
AVA	Aneuploidy, animal cells <i>in vivo</i>
CBA	Chromosomal aberrations, animal bone-marrow cells <i>in vivo</i>
CCC	Chromosomal aberrations, spermatocytes treated <i>in vivo</i> , spermatocytes observed
CGC	Chromosomal aberrations, spermatogonia treated <i>in vivo</i> , spermatocytes observed
CGG	Chromosomal aberrations, spermatogonia treated <i>in vivo</i> , spermatogonia observed
CHL	Chromosomal aberrations, human lymphocyte <i>in vitro</i>
CIC	Chromosomal aberrations, Chinese hamster cells <i>in vitro</i>
CIR	Chromosomal aberrations, rat cells <i>in vitro</i>
CLH	Chromosomal aberrations, human lymphocytes <i>in vivo</i>
COE	Chromosomal aberrations, oocytes or embryos treated <i>in vivo</i>
DLM	Dominant lethal test, mice
DLR	Dominant lethal test, rats
DLT	Dominant lethal test (Gene-Tox)
DMM	<i>Drosophila melanogaster</i> , somatic mutation (and recombination)
DMN	<i>Drosophila melanogaster</i> , aneuploidy
DMX	<i>Drosophila melanogaster</i> , sex-linked recessive lethal test
ECW	<i>Escherichia coli</i> WP2 uvrA, reverse mutation
GIA	Gene mutation, other animal cells <i>in vitro</i>
GIH	Gene mutation, human cells <i>in vitro</i>
G5T	Gene mutation, mouse lymphoma L5178Y cells, <i>tk</i> locus
G51	Gene mutation, mouse lymphoma L5178Y cells, all other loci
G9H	Gene mutation, Chinese hamster lung V-79 cells, <i>hprt</i> locus
G9O	Gene mutation, Chinese hamster lung V-79 cells, ouabain resistance
MHT	Mouse heritable translocation test
MIA	Micronucleus test, animal cells <i>in vitro</i>
MST	Mouse spot test
MVM	Micronucleus test, mice <i>in vivo</i>
SA0	<i>Salmonella typhimurium</i> TA100, reverse mutation
SA5	<i>Salmonella typhimurium</i> TA1535, reverse mutation
SA7	<i>Salmonella typhimurium</i> TA1537, reverse mutation
SA8	<i>Salmonella typhimurium</i> TA1538, reverse mutation
SA9	<i>Salmonella typhimurium</i> TA98, reverse mutation
SCN	<i>Saccharomyces cerevisiae</i> , aneuploidy
SCR	<i>Saccharomyces cerevisiae</i> , reverse mutation
SHL	Sister chromatid exchange, human lymphocytes <i>in vitro</i>
SIC	Sister chromatid exchange, Chinese hamster cells <i>in vitro</i>
SIR	Sister chromatid exchange, rat cells <i>in vitro</i>
SLH	Sister chromatid exchange, human lymphocytes <i>in vivo</i>
SLP	Mouse specific locus test, post-spermatogonial
SLO	Mouse specific locus test, other stages
SLT	Mouse specific locus test (Gene-Tox)
SVA	Sister chromatid exchange, animal cells <i>in vivo</i>
TBM	Cell transformation, BALB/c 3T3 mouse cells
TCM	Cell transformation, C3H 10T1/2 mouse cells
TCS	Cell transformation, Syrian hamster embryo cells, clonal assay
TRR	Cell transformation, RLV/Fischer rat embryo cells

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## REFERENCES

1. Brockman, H. E., and DeMarini, D. M. Utility of short-term tests for genetic toxicity in the aftermath of the NTP's analysis of 73 chemicals. *Environ. Molecular Mutagen.* 11: 421-435 (1988).
2. Ashby, J., and Morrod, R. S. Detection of human carcinogens. *Nature* 352: 185-186 (1991).
3. Ashby, J., and Tennant, R. W. Chemical structure, Salmonella mutagenicity and extent of carcinogenicity as indicators of genotoxic carcinogenesis among 222 chemicals tested in rodents by the U.S. NCI/NTP. *Mutat. Res.* 204: 17-115 (1988).
4. Butterworth, B. E. Consideration of both genotoxic and mechanisms in predicting carcinogenic potential. *Mutat. Res.* 239: 117-132 (1990).
5. Ashby, J., Tennant, R. W., Zeiger, E., and Stasiewicz, S. Classification according to chemical structure, mutagenicity to Salmonella and level of carcinogenicity of a further 42 chemicals tested for carcinogenicity by the U.S. National Toxicology Program. *Mutat. Res.* 223: 73-103 (1989).
6. Bartsch, H., and Malaveille, C. Prevalence of genotoxic chemicals among animal and human carcinogens evaluated in the IARC Monograph series. *Cell Biol. Toxicol.* 5: 115-127 (1989).
7. Moody, D. E., Reddy, J. K., Lake, B. G., Popp, J. A., and Reese, D. H. Peroxisome proliferation and nongenotoxic carcinogenesis: commentary on symposium. *Fundam. Appl. Toxicol.* 16: 233-248 (1991).
8. Waters, M. D., and Auletta, A. The Gene-Tox program: genetic activity evaluation. *J. Chem. Info. Computer Sci.* 21: 35-38 (1981).
9. Waters, M. D., Stack, H. F., Brady, A. L., Lohman, P. H. M., Haroun, L., and Vainio, H. Use of computerized data listings and activity profiles of genetic and related effects in the review of 195 compounds. *Mutation Res.* 205: 294-312 (1988).
10. Rinchik, E. J., Bangham, J. W., Hunsicker, P. R., Cacheiro, N. L. A., Kwon, B. S., Jackson, I. J., and Russell, L. B. Genetic and molecular analysis of chlorambucil-induced germ-line mutations in the mouse. *Proc. Natl. Acad. Sci. U.S.A.* 87: 1416-1420 (1990).
11. Anderson, D., McGregor, D. B., Purchase, I. F. H., Hodge, M. C. E., and Cuthbert, J. A. Dominant-lethal test results with known mutagens in two laboratories. *Mutat. Res.* 43: 231-246 (1977).
12. Russell, L. B., Hunsicker, P. R., Cacheiro, N. L. A., and Generoso, W. M. Induction of specific locus mutations in male germ cells of the mouse by acrylamide monomer. *Mutat. Res.* 262: 101-107 (1991).
13. Adler, I.-D., Ingwersen, I., Kliesch, U., and El Tarras, A. Clastogenic effects of acrylamide in mouse bone marrow cells. *Mutat. Res.* 206: 379-385 (1988).
14. Cihak, R., and Vontorkova, M. Cytogenetic effects of acrylamide in the bone marrow of mice. *Mutat. Res.* 209: 91-94 (1988).
15. Adler, I.-D., and Ashby, J. The present lack of evidence for unique rodent germ-cell mutagens. *Mutat. Res.* 212: 55-66 (1990).
16. Obe, G., and Anderson, D. Genetic effects of ethanol. *Mutat. Res.* 186: 177-200 (1987).
17. Mavournin, K. H., Blakey, D. H., Cimino, M. C., Salamone, M. F., and Heddle, J. A. The *in vivo* micronucleus assay in mammalian bone marrow and peripheral blood. A report of the U.S. Environmental Protection Agency Gene-Tox Program. *Mutat. Res.* 239: 29-80 (1990).
18. Tennant, R. W., and Ashby, J. Classification according to chemical structure, mutagenicity to Salmonella and level of carcinogenicity of a further 39 chemicals tested for carcinogenicity by the U.S. National Toxicology Program. *Mutat. Res.* 257: 209-227 (1991).
19. Dearfield, K. L., Auletta, A. E., Cimino, M. C., and Moore, M. M. Considerations in the U.S. Environmental Protection Agency's testing approach for mutagenicity. *Mutat. Res.* 258: 259-283 (1991).
20. Ashby, J. The prospects for a simplified and internationally harmonized approach to the detection of possible human carcinogens and mutagens. *Mutagenesis* 1: 3-16 (1986).
21. Arnold, D. L., Moodie, C. A., Grice, H. C., Charbonneau, S. M., Stavric, B., Collins, B. T., McGuire, P. F., Zawidzka, Z. Z., and Munro, I. C. Long-term toxicity of ortho-toluenesulfonamide and sodium saccharin in the rat. *Toxicol. Appl. Pharmacol.* 52: 113-152 (1980).
22. Elcombe, C. R., and Mitchell, A. M. Peroxisome proliferation due to di(2-ethylhexyl)phthalate (DEHP): species differences and possible mechanisms. *Environ. Health Perspect.* 70: 211-219 (1986).
23. Eacho, P. I., Foxworthy, P. S., Johnson, W. D., Hoover, D. M., and White, S. L. Hepatic peroxisomal changes induced by tetrazole-substituted alkoxyacetophenone in rats and comparison with other species. *Toxicol. Appl. Pharmacol.* 83: 430-437 (1986).
24. Reddy, J. K., Lalwani, N. D., Qureshi, S. A., Reddy, M. K., and Moehle, C. M. Induction of hepatic peroxisome proliferation in nonrodent species, including primates. *Amer. J. Pathol.* 114: 171-183 (1984).
25. Lake, B. G., Evans, J. G., Gray, T. J. B., Korsosi, S. A., and North, C. J. Comparative studies on nafenopin-induced hepatic peroxisome proliferation in the rat, Syrian hamster, guinea pig, and marmoset. *Toxicol. Appl. Pharmacol.* 99: 148-160 (1989).
26. Butterworth, B. E. Nongenotoxic carcinogens. *CIIT Activities* 7: 1-6 (1987).
27. NTP. Toxicology and Carcinogenesis Studies Technical Reports. National Toxicology Program, Research Triangle Park, NC, 1977-1988.
28. IARC. Monographs on the Evaluation of Cancer in Humans. International Agency for Research on Cancer, Lyon, 1985-1992.